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13. ABSTRACT ***(Maximum 200 Words)***

Neuronal death occurs in the brain during development and in pathological conditions, like Alzheimer's disease and stroke. Tissue plasminogen activator (tPA), a protease converting plasminogen to plasmin, is necessary for neurodegeneration. In mice lacking tPA ($tPA^{-/-}$), neurons are resistant to neurotoxic death. Delivery of tPA into $tPA^{-/-}$ mice restores susceptibility to neuronal death, indicating that tPA is neurotoxic in the context of excitotoxic injury. Although tPA is synthesized by neurons, the increase in tPA upon injury derives primarily from activated microglia, the immune cells of the brain. Microglia in $tPA^{-/-}$ mice demonstrate reduced activation. Using tPA as tool, we are determining whether microglia are neuroprotective or neurotoxic, and what are the cell types involved in the sequence of events that lead from injury to neuronal death. We have established primary cultures and obtained information from them on the source of tPA that initiates neurotoxin-induced cell death (Spec. aim 2), and have narrowed the region of tPA that promotes microglial activation (Spec. aim 1). Since exaggerated neurodegeneration is evident in pathological conditions, understanding the underlying mechanisms could prove beneficial for interfering with the pathologies.

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Styliani-Anna E. Tsirka

PI - Signature 7/19/00 Date

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Introduction

The application entitled "Tissue plasminogen activator (tPA) mediates neurotoxin-induced cell death and microglial activation" proposes to address 2 questions / objectives.

1. **How does tPA mediate microglial activation? Does the activation involve classical signal transduction pathways, namely tyrosine phosphorylation of a specific receptor and a cascade of activation of kinases, leading ultimately to transcriptional upregulation of critical immediately early or early genes? How does modulation of tPA / microglial activation affect neurotoxicity?**

We are in the process of defining the mechanism by which tPA mediates microglial activation using deletion mutants and eventually single point mutants, first in a cell culture system and then in vivo, in mice. Furthermore, several classical pathways of signal transduction are evaluated, starting with the MAP-kinases pathway.

2. **What is the source(s) of tPA that mediates microglial activation and neuronal degeneration in response to excitotoxin-stimulation in culture?**

We have propose to mix-and-match neuronal and microglial cultures from wild-type and tPA-deficient genotypes in the presence of excitotoxins and investigate the progress and mechanism by which neuronal death proceeds. We have established the mixing-and-matching protocols

Body

Objective 1) Which domain of tPA is required for microglial activation?

Tissue plasminogen activator (tPA), a blood clot dissolving enzyme, is a serine protease that converts inactive plasminogen (plg) to the active protease plasmin, and thus initiates a potent proteolytic cascade. Catalytically-inactive tPA mediates microglial activation, implying that non-proteolytic domains in tPA act to trigger a microglial signaling pathway (Rogove et al., 1999). The tPA protein, besides its catalytic, serine-protease domain at the carboxy-terminus, contains additional structures, including a finger domain, an EGF-like domain, and two kringle motifs, thought to participate in protein-protein interactions. To investigate the role of tPA in microglial activation, primary mixed and pure microglial cultures were used. The four non-protease structural domains of tPA (finger, growth-factor-like, kringle 1, and kringle 2) will be tested individually. We engineered the deletion mutants in the lab. All the mutants were constructed.

Mutant 1 (Δfinger) was made by PCR by directly introducing an ATG at the 5' of the growth factor domain. Mutants 2 (ΔGrowth Factor),3 (ΔKringle1), and 4 (ΔKringle 2) were generated using PCR: the relevant domain(s) were deleted (looped out). The deletion mutants were confirmed by sequencing. All the mutants, were cloned into the pCGN vector (tPA expression is controlled by the CMV promoter), and were co-transfected in HeLa cells, along with pMC1neo, and stable transformants will be selected. Each deletion-mutant form of tPA were purified through a heparin-agarose column, and the purity confirmed by SDS-PAGE. The purified deletion mutants were added individually to $tPA^{-/-}$ microglia and were tested for their ability to restore full LPS-induced microglial activation. The level of microglial activation will be assessed by morphology, evaluation of the levels of F4/80 by western analysis, and measurement of production of NO. Wild-type and $tPA^{-/-}$ microglia treated accordingly served as controls for setting the upper and lower limits in the NO measurements. Our preliminary data suggest that it

is the Finger domain that binds to microglial cell surface and promotes microglial activation: When the ΔF mutant is added to LPS-stimulated $tPA^{-/-}$ microglia the levels of F4/80 microglial marker, and the levels of NO produced remain as low as the non-LPS treated microglia (see Figs. 1 and 2). Furthermore, addition of the ΔGF mutant resulted in over-stimulation of the LPS-treated $tPA^{-/-}$ microglia (see Figs. 1 and 2), suggesting that this domain normally may act to regulate binding and microglial activation confered by the Finger domain.

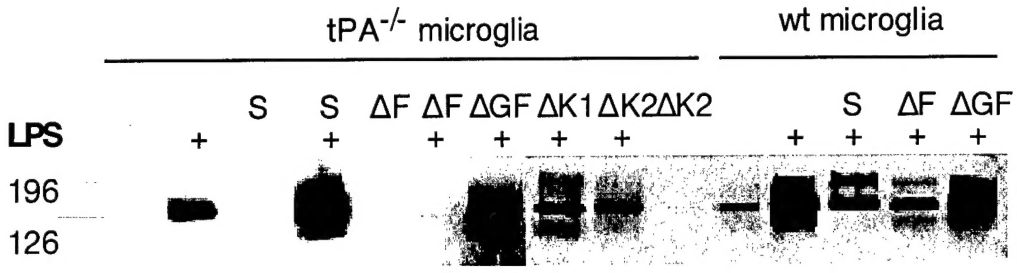

Figure 1: We stern blot analysis of the microglial marker F4/80 expression in primary wild-type (wt) or tPA-deficient ($tPA^{-/-}$) microglia cultures in the absence or presence (+) of LPS stimulation. Various tPA mutants have been added to the cultures. S: S478AtPA; ΔF: tPA lacking the finger domain; ΔGF: tPA lacking the growth factor domain; ΔK1: tPA lacking the kringle 1 domain; ΔK2: tPa lacking the kringle 2 domain.

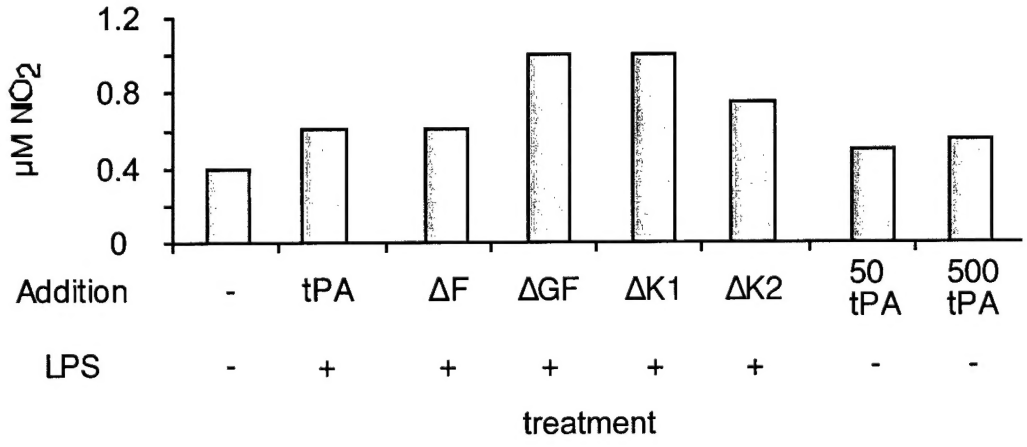

Figure 2: Production of NO in tPA-deficient microglia in the presence (+) or absence (-) of LPS stimulation after the addition of either recombinant tPA of tPA delection domain mutants.

The ΔF tPA domain will be analyzed further by evaluating single amino acid mutations within the domain in the primary cultures as above. Furthermore we will investigate extensively the contribution of the growth factor domain in microglial activation.

Specific mutants that fail to activate microglia in culture will be tested in vivo: The mutant(s) will be infused into the hippocampus of $tPA^{-/-}$ mice which will then be subjected to unilateral injection of neurotoxins. Neuronal survival and the status of microglial activation will be estimated as described above. Of particular interest will be any mutant tPA molecule(s) that retain catalytic activity but are unable to activate microglia. Infusion of these mutants will allow us to determine whether tPA-mediated neurodegeneration can be achieved in the absence of microglial activation.

We have also started evaluating the signal transduction cascades induced in microglia that result from tPA-mediated microglial activation. Our preliminary evidence suggests that probably the JAK/Stat pathway would be involved, since upon tPA-mediated microglial activation, STAT5a and 5b become up-regulated. We will continue to investigate the pathway of mciroglial activation, n order to obtain critical check-points in the process, that would allow us to regulate accordingly neuronal degeneration.

Objective 2) What is the source(s) of tPA that mediates microglial activation and neuronal degeneration in response to excitotoxin-stimulation in culture?

tPA is synthesized both by neurons and microglia, whereas plasminogen is synthesized exclusively by neurons. When the excitotoxic insult occurs, glutamate receptor-expressing neuronal cells become activated, secrete neurotransmitters and initiate the sequence of events that result in cell death. With respect to tPA, during excitotoxic injury up-regulation of its expression is observed, both in microglial and neuronal cells. tPA can then mediate microglial activation, and also act on plasminogen to promote neurodegeneration. Although both neurons and microglia secrete tPA into the extracellular space, it is not known whether tPA from the two sources undertakes similar or different functions. For example, neuronal tPA might cause neuronal degeneration, whereas microglial tPA might function only to activate microglia to carry out phagocytosis and removal of neuronal debris. To determine the functional role for tPA for each source in the hippocampus, we are using primary cultures from wild-type or $tPA^{-/-}$-derived microglia and neurons to identify the specific function of tPA from each cell type.

Hippocampal neurons from $tPA^{-/-}$ mice are plated and maintained in the absence of any other cell type. Pure microglial cells from wild-type mice are then mixed with the neuronal cultures, and low concentrations of glutamate (25 μM, to ascertain that only apoptotic cell death takes place) is added to the cultures. If microglial tPA is the initiating factor for glutamate-induced neuronal death, then the neurons will undergo apoptosis. If, however, neurons are the source of the effective tPA, then at best delayed death will be observed. If the changes that occur in neurons after the excitotoxic insult induce microglial activation, the added wild-type microglia will proliferate and undergo morphological changes that will be monitored by F4/80 immunostaining.

The converse experiment involves mixing wild-type hippocampal neurons with $tPA^{-/-}$-microglia (and subsequent addition of glutamate). If neuronal tPA initiates the cell death cascade, then the tPA produced by the neurons will be enough to promote degeneration; if, however, the major player is microglial tPA, then persistence of neurons will be observed.

Microglial activation was evaluated by western blot analysis of the microglial marker F4/80 (see Figure 3).

The degree of neuronal survival was evaluated by morphology, and by staining with trypan blue(see Figure 4), and by immunostaining for neuronal markers. In addition, the levels of LDH released are determined (as an indicator of neuronal death).

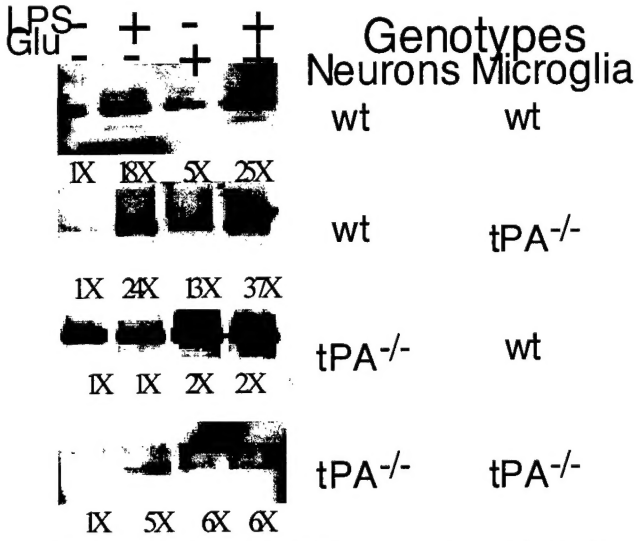

Figure 3: Western blot analysis of F4/80 expressing microglia after a 'mixing-and-matching' experiment. Neurons were exposed to 25 μM glutamate prior to exposure to microglia, and microglia were stimulated with 100 nM LPS prior to the addition to the neurons

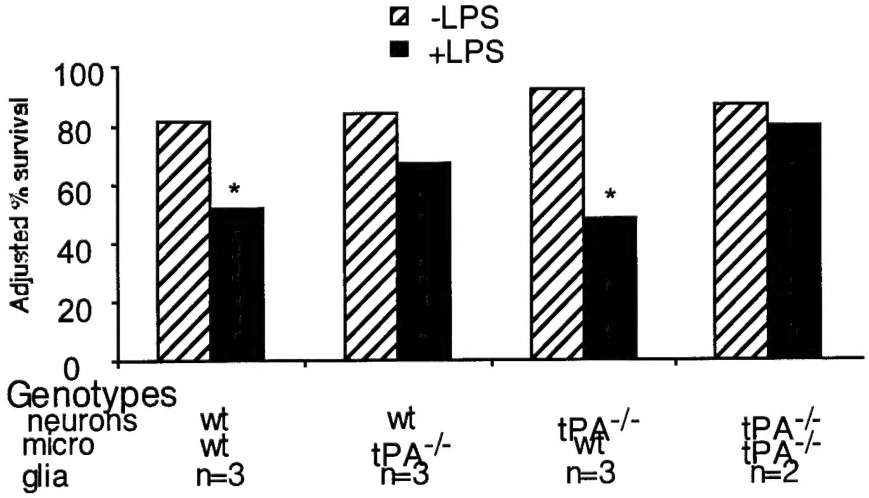

Figure 4: Neuronal survival estimated by trypan blue cell counts of experiments performed as described in Fig 3.

From the experiments described above we can draw the conclusion that although neuronal tPA promotes microglial activation, it is microglial tPA production that contributes the most to microglial activation (in an autocrine fashion).

As far as neuronal death is concerned, microglial tPA appears to be the major player as well, making evident that understanding microglial function in the context of neurotoxic death is crucial, since it may allow us to interfere with this form of cell death.

Key Research Accomplishments

- Narrowed down the site of interaction between tPA and microglial cells. It is the finger domain
- Defined that the growth factor-like domain regulates the interaction, possibly acting as a dominant negative
- Determined that neuronal tPA is not sufficient to mediate neurotoxin-induced cell death, but needs the presence of higher concentrations of microglial tPA
- Determined that lipopolysaccharide-induced microglial activation and excitotoxin-induced microglial activation proceed via different signaling pathways

Reportable Outcomes

A. Manuscript:
A.D. Rogove, W. Lu, S. Strickland and S.E. Tsirka. "Excitotoxic stimulation of *op/op* mice reveals that only a small number of microglia are required to elicit neurodegeneration", Neuroscience, *submitted.*

B. Abstracts:

- CJ Siao, AD Rogove and SE Tsirka. "Neuronal- and microglial-specific tissue plasminogen activator play different but complementary roles in excitotoxicity-induced neurodegeneration in the mouse", IV European Meeting on Glial cell function in Health and Disease, May 24-27 2000, Barcelona, Spain.
- AD Rogove, W Lu, and SE Tsirka. "Kainate-induced neuronal cell death in op/op mice points to the presence of a necessary and sufficient number of activated microglia", IV European Meeting on Glial cell function in Health and Disease, May 24-27 2000, Barcelona, Spain.

Conclusions

The major findings of our funded research up to now point to the finger domain of tPA as the domain responsible for binding and thus mediating microglial activation. We are in the process of making single point mutations to narrow down the specific site of interaction. Knowing the binding domain will allow us to use it as a bait to identify the receptor for tPA on microglial

cells, and thus generate a reagent very useful for turning on and off microglial activation. Such an accomplishment will allow us to modulate the neurotoxic effects of microglial activation which is involved in promoting neuronal death in various neurodegenerative diseases.

References

Rogove, A., Siao, C.-J., Keyt, B., Strickland, S., and Tsirka, S. (1999). Activation of microglia reveals a non-proteolytic cytokine function for tissue plasminogen activator in the central nervous system, J Cell Sci *112*, 4007-4016.

Excitotoxic stimulation of *op/op* mice reveals that only a small number of microglia are required to elicit neurodegeneration

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Running title: Microglia possess strong neurotoxic properties

Abstract

Microglial proliferation and activation occurs concurrently with excitotoxin-induced neurodegeneration. We previously reported that microglia can exhibit neurotoxic behaviors after injection of excitotoxins into the murine hippocampus. It has not been known whether microglial proliferation is critically involved in neurodegeneration, or whether activation of the pre-existing resident microglia may suffice. Using osteopetrotic (*op/op*) mice, in which microglial proliferation does not occur, we demonstrate here that only small numbers of activated microglia are sufficient to promote neurodegeneration. Our data suggest that a threshold exists above which a maximal microglial contribution to neurotoxicity is observed. This threshold appears to be sufficiently low, so that only approximately 40% of the numbers of microglia present in wild-type mice need to become activated to contribute to neurodegeneration. Furthermore, since this decrease in microglial numbers coincides with a similar decrease in tissue plasminogen activator's enzymatic activity, we suggest that tissue plasminogen activator can be used as a marker of microglial proliferation.

Key Words: microglia, tissue plasminogen activator, neurotoxicity, osteopetrotic mouse.

Introduction

When an excitotoxin is introduced into the mammalian hippocampus, neuronal death soon follows. This neuronal injury is accompanied by activation of the resident microglial cells[1]. Microglial activation is followed by their migration to the site of injury, local proliferation, changes in gene expression, presentation of class II major histocompatibility antigens, and phagocytosis. We and others have shown that microglia can exhibit neurotoxic properties when neuronal injuries are elicited by local injections of excitotoxins[1, 15], autoimmune inflammation[12], or ischemia[20, 23]. Cytokines and neurotoxins secreted by microglia may help regulate the responses to injury in the central nervous system[8, 9, 19].

In mice deficient for tissue plasminogen activator (tPA), a secreted serine protease normally present in the brain, hippocampal neurons are resistant to excitotoxic glutamate analogues and the microglia display attenuated activation[16]. This attenuation is evident both as a decrease in the number of activated microglia present and as a decrease in the levels of expression of various microglial markers and cytokines[14]. Additional evidence suggests that tPA generated by microglia contributes to the neurotoxicity[15]. We wanted accordingly to evaluate if a decrease in the number of activated microglia reduced excitotoxic neural injury, and thus set out to ask this question using the mutant osteopetrotic (*op/op*) mouse.

Op/op is a spontaneous mutation in C57/Bl6 mice resulting in a deficiency of the macrophage colony stimulating factor (CSF-1) gene product. A single base pair insertion within this gene causes a frameshift mutation and creates a truncated protein that is non-functional[24]. In cell culture, microglia isolated from *op/op* mice are unable to proliferate unless the medium is supplemented with CSF-1[4]. Furthermore, microglial proliferation is dramatically reduced in the nucleus of the transected facial nerve after axotomy in *op/op* mice[13]. In contrast, Witmer-Pack et al. reported that microglial cells are not completely dependent on CSF-1, although nonetheless, the number of microglia in the brain is significantly decreased[22]. According to a recent report,

there are approximately 34-47% fewer microglial cells in *op/op* brains in the frontal and parietal cortex and corpus collosum regions than in wild-type brains[21].

To investigate whether decreasing the number of microglial cells ameliorates neuronal survival after excitotoxic injury, we injected kainic acid unilaterally into the brains of control (C57/Bl6 or *op/+*) and *op/op* mice. In this report we show that microglial activation is normal after excitotoxic injury in *op/op* mice, but microglial proliferation is deficient. Furthermore, the neuronal death that follows excitotoxic injury in wild-type mice similarly occurs in *op/op* mice, suggesting that the activation of microglia rather than their proliferation is the critical event. Additionally, the levels of tPA in the kainate-injected brains of C57/Bl6 and *op/op* mice correlated well with the amount of activated microglia present, suggesting that tPA may be a useful marker for microglial proliferation. Taken together with our previous finding that inhibition of microglial activation protects from neurodegeneration, we conclude here that even approximately half the numbers of microglia suffice to promote excitotoxin-mediated neurodegeneration.

Experimental Procedures

Animal procedures: All experiments performed on mice were done in accordance to the NIH guide for the care and use of laboratory animals as well as the institutional guidelines set by the Division of Laboratory Animal Research at Stony Brook. All efforts were made to minimize the use of animals and to ensure minimal suffering of those animals used.

Mice: The osteopetrosis (*op/op*) mouse arose form a spontaneous mutation in the CSF-1 gene in C57/ Bl6 mice. This single base insertion within the coding region of CSF-1 leads to a truncated, non-functional protein[24]. Since homozygote *op/op* mice do not breed well, *op/+* heterozygotes were mated to expand the *op/op* colony. The genotypes of the offspring were determined using a PCR-based assay. Primers that flanked the mutation were designed (primer 1: 5'-CAGCTGGATGATCCTGTTTGC-3'; primer 2: 5'-CTCGGTGGCGTTAGCATTGGG-3') such that genomic DNA from a homozygote *op/op* mutant would yield a 111 base pair product whereas wild-type DNA would yield a 110 base pair product. Genomic DNA was prepared from the tails of mice using standard procedures. The genomic DNA was PCR-amplified using the above mentioned primers using the following protocol: 94°C 5 min followed by 30 cycles of 94°C 45 sec, 58°C 1 min. 72°C 1 min, with a final extension at 72°C for 7 minutes. ^{32}P-dATP was included in the PCR reactions. The products were separated on a 6% polyacrylamide gel and subjected to autoradiography to visualize the bands (Fig. 1).

Intrahippocampal injection of kainate: Adult C57Bl6/J, *op/+* and *op/op* male mice, between 20 and 25 grams, were injected intraperitoneally with atropine (0.6 mg/kg of body weight) and deeply anesthetized with 2.5% avertin (0.02 ml/gram of body weight). The mice were then injected with 1.5 nmol kainic acid (in 300 nl phosphate buffered saline) unilaterally into the hippocampus using stereotaxic coordinates (bregma -2.5 mm, medial/lateral 1.7 mm and dorsoventral 1.6 mm). The excitotoxin was delivered over 30 sec, and the injection needle remained in place for two additional minutes to prevent reflux of fluid. Five days after the injection, the brains of the injected mice were analyzed for neuronal survival and microglial activation.

Immunohistochemistry: Coronal sections (30 μm) of the brains of the injected mice were cut at the level of the hippocampus. The sections were incubated with antibodies either to the mature macrophage/microglia specific antigen F4/80 (1:100, Serotec) or Mac-1 (1:10, Roche Biochemicals). Biotinylated secondary antibodies were used (Vector Laboratories) and the avidin-biotin-peroxidase complex (ABC reaction) was visualized with diaminobenzidine and hydrogen peroxide (Vector Laboratories) as described previously[17].

Amidolytic assay for tPA activity: For quantitative determination of tPA activity, the amidolytic assay was performed as previously described[2]. Briefly, the tissue was lysed in 0.25% Triton X-100 and incubated at 25°C in a mix containing 0.3 mM S-2251 and 0.42 μM plasminogen in 0.1 M Tris, pH 8.1, 0.1% Tween-80). The change in absorbence (ΔA) at 405 nm was measured at different time-points. Known concentrations of recombinant tPA protein were used to generate a standard curve.

Quantitation of microglial cell numbers: $F4/80^+$ and $Mac\text{-}1^+$ microglia in the CA1 hippocampal subfield were counted in four successive sections around the injection site, and the numbers were averaged, as described[3]. Microglial cell counting occurred five days after the excitotoxic injury, when microglial activation (evident by F4/80 immunostaining) reaches peak levels[1]. Activated microglia were characterized as cells with a cell body larger than 10 μm in diameter, with short, thick processes and intense immunoreactivity. No resting microglia (characterized by small cell body, long processes and weak immunoreactivity) were observed at that time-point on the sections.

Quantification of microglial activation: Whole brain lysates were prepared by dounce homogenization in phosphate buffered saline from kainate-injected control and *op/op* mice. To estimate the relative amounts of activated microglia present in each brain, relative tumor necrosis factor alpha (TNF-α) levels were assessed by Western blot analysis. Briefly 20 μg of total lysate was separated through a 15% polyacrylamide gel and transferred to a PDVF membrane. TNF-α was detected using a rat-anti-mouse TNF-α antibody (clone MP6-XT3) at a dilution of 1:500 (Boehringer Mannheim) followed by biotinylated goat-anti-rat secondary antibody at a 1:3000 dilution (Vector Laboratories). Finally, the avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories) was visualized by chemiluminescence (LumiGLO, KPL). The relative amounts of TNF-α were determined using a Bio-Rad densitometer.

Results

Decreased proliferation, but wild-type-like morphological activation of *op/op* microglia after kainic acid injection

After injection of kainic acid into the hippocampus of wild-type mice, microglial cells undergo activation. The kinetics of this activation process have been determined[1]. The maximum levels of microglial activation, as evidenced by dramatic morphological changes, are reached between five and fourteen days after the injection[1]. We evaluated the levels of microglial activation in *op/op* mice five days after kainate injection by immunohistochemistry with the monoclonal antibodies F4/80 (Fig. 2) and Mac-1 (data not shown). Microglia in the *op/op* mice acquire the morphological characteristics of activation after kainate injection (compare panels 2E and 2F). However, fewer activated microglia are observed in the hippocampus of *op/op* mice than in

control mice (see Table). To further assess the relative quantity of activated microglia in *op/op* and control mice we evaluated the levels of TNF-α present in brain lysates from each genotype by western blot analysis (data not shown). TNF-α is a cytokine that is produced by microglia as part of the activation process. Kainate-injected brain lysates from *op/op* mice contained 48% of the amount of TNF-α present in lysates from control mice. These quantitative data agree with the qualitative observation that fewer microglia are present in *op/op* mice.

The migration of microglia in the *op/op* mice to the injury site is comparable to that of microglia in control mice (note the presence of activated microglia in the CA1 hippocampal subfield mainly in the injected sides of both mice, Fig. 2A and B). Even at higher magnifications, the microglia in control and *op/op* mice appear similar aside from minor morphological differences visualized using F4/80 staining as reported previously[21]. Therefore, in this excitotoxin-injection model, microglia in *op/op* mice display the phenotypic characteristics of microglial activation but do not mount a proliferative response at the site of injury (as evident by the presence of fewer activated F4/80$^+$ cells). This observation is in agreement with the data obtained in the facial nerve axotomy model [13].

The pyramidal neurons in *op/op* mice are susceptible to excitotoxic cell death.

We have previously shown that microglial activation is significantly attenuated following kainate injection in tPA-deficient mice, with respect to changes in morphology[16]. Furthermore, the pyramidal neurons in these mice, as well as in wild-type mice in which microglial activation has been delayed by macrophage-microglial inhibitory factor[15], appear resistant to excitotoxin-induced neuronal death. To evaluate whether the absence of the proliferative microglial response was sufficient to confer protection against neuronal cell death, we assessed neuronal survival in control (*op/+*), and *op/op* mice after the unilateral injection of kainate. As seen in Figure 3, the pyramidal hippocampal neurons were sensitive to excitotoxin injection in both genotypes, suggesting that there were enough activated microglia present in *op/op* mice to mediate neuronal death.

Decreased levels of tissue plasminogen activator in *op/op* mice.

We have previously shown that tPA can promote excitotoxic cell death in the mouse brain and suggested that secreted microglial-derived tPA may contribute to the neurotoxic properties of these cells[15]. Given the lower number of microglial cells present in *op/op* mice, we performed both zymographic and amidolytic assays to determine the levels of tPA. As shown in the Table, there is a significant difference between the levels of tPA in the kainate-injected brains of control mice (4.63 ng of active tPA / μg of brain extract), and *op/op* mice (1.99 ng of active tPA / μg of brain extract) as demonstrated by the amidolytic assay. Similar differences in tPA levels were noted by zymographic analysis (data not shown). It is interesting to note in the *op/op* forebrain there is ~43% tPA activity present compared to that of wild-type mice, a number close to the difference in activated microglia numbers between the two genotypes (see the Table). This result suggests that tPA may be a marker for microglial proliferation in the mouse forebrain.

Discussion

The *op/op* mouse carries a frameshift in the CSF-1 gene[24] that results in defective microglial proliferation both in cell culture and in vivo[4, 13]. Previously, we reported that retarding microglial activation in response to excitotoxin injection could protect against neuronal death[15]. This

suggested a neurotoxic role for microglia in the mouse brain during excitotoxicity. However, it was uncertain whether the proliferation of microglia was also necessary for excitotoxin-mediated cell death. We set out therefore to ascertain whether impairment of microglial proliferation would confer protection from neuronal death, or if few activated microglia would suffice to promote neuronal death. Intrahippocampal delivery of kainic acid into the brain parenchyma of the *op/op* mice resulted in neuronal cell death. These results suggest that despite the diminished proliferative capability of microglia in *op/op* mice, the microglia that become activated are able to promote neurotoxicity and to phagocytose the debris of injured neuronal cells. In addition it is suggested that a threshold may exist for the number of activated microglial cells above which they cease to become a rate-limiting factor in this neurodegeneration pathway.

These neurotoxic properties of microglia may be the result of the up-regulation and secretion of several factors that these cells express, such as tPA and TNF-α[10, 14]. Interestingly, at least TNF-α (and potentially tPA) lies at the beginning of potent signal cascades that can result in cell death [5]. This provides a rationale through which even a small number of activated microglia could provide the necessary downstream signals to effect neuronal cell death. We have already shown that the tPA/plasminogen proteolytic cascade promotes excitotoxic cell death[17, 18]. Furthermore, microglial-derived tPA may initiate this cascade (C.-J. Siao, personal communication). The data presented here demonstrate that the number of activated microglia is diminished in the kainate-injected *op/op* mouse (39% of control), and these data correlate with the levels of decreased enzymatic activity of tPA in kainate-injected *op/op* mice (43% compared to those of wild-type mice). This result further suggests that microglial tPA is a major source of tPA locally at the site of injury. The potential for signal amplification present in a proteolytic cascade initiated by tPA and plasmin may account for the strength of the neurotoxic effects of the few activated microglia in the *op/op* mouse. Moreover, we propose that tPA can be used as a marker for microglial proliferation following injury induced by kainate.

Additionally, TNF-α has been implicated to play a role in the neurotoxic properties which microglia possess[6, 25]. TNF-α is secreted by activated microglia and can inhibit the re-uptake of glutamate by astrocytes, thereby allowing higher and potentially toxic concentrations of extracellular glutamate[6]. TNF-α lies at the beginning of a biochemical cascade that potentiates neuronal death following kainate injection[5]. In fact, we report here that there is approximately half the level of TNF-α present in the kainate-injected *op/op* mouse, yet excitotoxin-induced neurodegeneration proceeds normally. This may occur because TNF-α can stimulate the generation of IL-1β, IL-6 and other cytotoxic cytokines[11]. However, we have also previously shown that in tPA-deficient mice (where microglial activation is attenuated[16]) neurons are resistant to excitotoxicity and the microglia in these mice secrete 43% less TNF-α than wild-type mice in response to activation stimuli[14]. This amount of TNF-α is comparable to that secreted by *op/op* microglia. It is tempting to speculate that the different effects of kainate in tPA-deficient and *op/op* mice lie in the ability of activated microglia in *op/op* mice to produce sufficient tPA to cross a threshold and successfully initiate the biochemical cascades that lead to cell death.

Activated microglia have been implicated to play a role in several neuropathological conditions such as Alzheimer's disease, stroke and multiple sclerosis. Previously, Raivich et al.[13] reported that microglia in *op/op* mice underwent normal activation in the facial nerve axotomy model. In the present study we show that the activation of microglia, rather than their proliferation, is

critical for effecting neuronal death. We and others previously demonstrated that microglia possess neurotoxic properties[7, 8, 15, 23]. Taken together with the data presented here, we suggest that the toxic mediators secreted by microglia are sufficiently potent that even decreased numbers of activated microglia are able to promote neuronal death. Therefore, any potential neuroprotective therapy involving suppression of microglial activation must take their potency into account.

Acknowledgements: We are grateful to Dr. M. Frohman for critical reading of the manuscript and the members of the Tsirka laboratory for helpful advice and discussions. This work was supported by fellowships from the MSTP program (A.D.R.), NIH (S.S. and S.E.T.) and an Army Medical Research Grant (S.E.T.).

Figure Legends

Figure 1: PCR-genotyping of *op/op* mice. Wild-type (C57/Bl6), *op*/+, and *op/op* mice were genotyped from tail DNA by an one-step PCR reaction as described in Materials and Methods. The PCR products were analyzed on a 6% polyacrylamide gel and visualized by autoradiography. C57/Bl6 mice amplify a single 110 base pair product (see arrow), *op*/+ mice amplify 110 and 111 base pair products, and the *op/op* mice amplify a single 111 base pair product (see arrow).

Figure 2: Microglial proliferation is deficient after excitotoxic injury, but microglial activation proceeds normally in *op/op* mice. Kainate was injected unilaterally into the hippocampi of control (*op*/+) and *op/op* mice to induce microglial responses (arrows indicate sites of injection on panels A and B). Control mice respond with activation of their microglia on the injected side (panels C and E). Such activation also includes a strong microglial proliferative response (seen best in panel C). While the proliferation of microglia in the injected side of the hippocampus of the *op/op* mice is defective (seen best in panel D), a higher powered photomicrograph indicates that the microglia that do migrate to the site of injury have the characteristics of activated microglia (panels D and F). The hippocampal subfields CA1, CA2 and CA3 as well as the dentate gyrus (DG) are labeled.

Figure 3: *Op/op* mice are sensitive to excitotoxin-induced neuronal death. The viability of pyramidal neurons in the hippocampus of injected control (*op*/+) and *op/op* mice after unilateral intrahippocampal kainate injection was evaluated by cresyl violet staining. In kainate-injected *op/op* mice (panel B), the neurons at the injected side were eliminated to the same extent as those in control (panel A) mice. The arrows indicate the site of kainate injection. CA1, CA2 and CA3 indicate the hippocampal pyramidal subfields, and DG denotes the dentate gyrus of the hippocampal formation.

Table: Activated microglia and tPA activity levels in the brains of *op / op* mice are diminished.

Activated microglia in the CA1 region of kainate-injected control (*op/+*) and *op/op* mice were counted as described in the text. Brains from control (C57/Bl6) and op/op mice were homogenized, as listed in the Experimental Procedures section, and the levels of tPA activity were measured by the amidolytic assay at 30 min, 90 min, 12 hrs and 24 hrs.

Genotype	Activated microglia in CA1*	tPA (ng/μg protein)**
Control	66.0 ± 6.78	4.63 ± 0.012
op / op	25.75 ±10.31	1.99 ± 0.206

* $p < 0.01$, value associated with Student's two-tailed t-test.

** $p < 0.05$, value associated with Student's two-tailed t-test.

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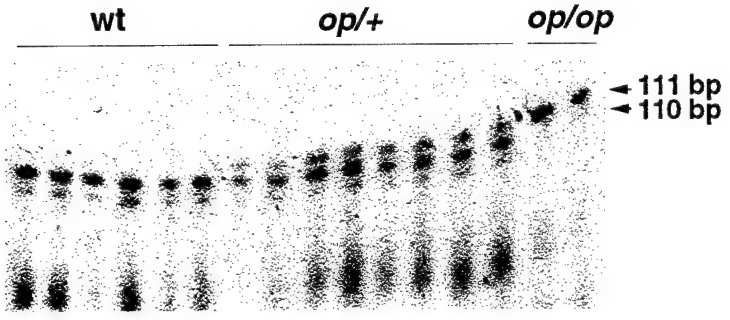
Abbreviations

CA, cornu ammonis
CSF-1, macrophage colony stimulating factor
IL, interleukin
op/op, osteopetrotic mouse
TNF, tumor necrosis factor
tPA, tissue plasminogen activator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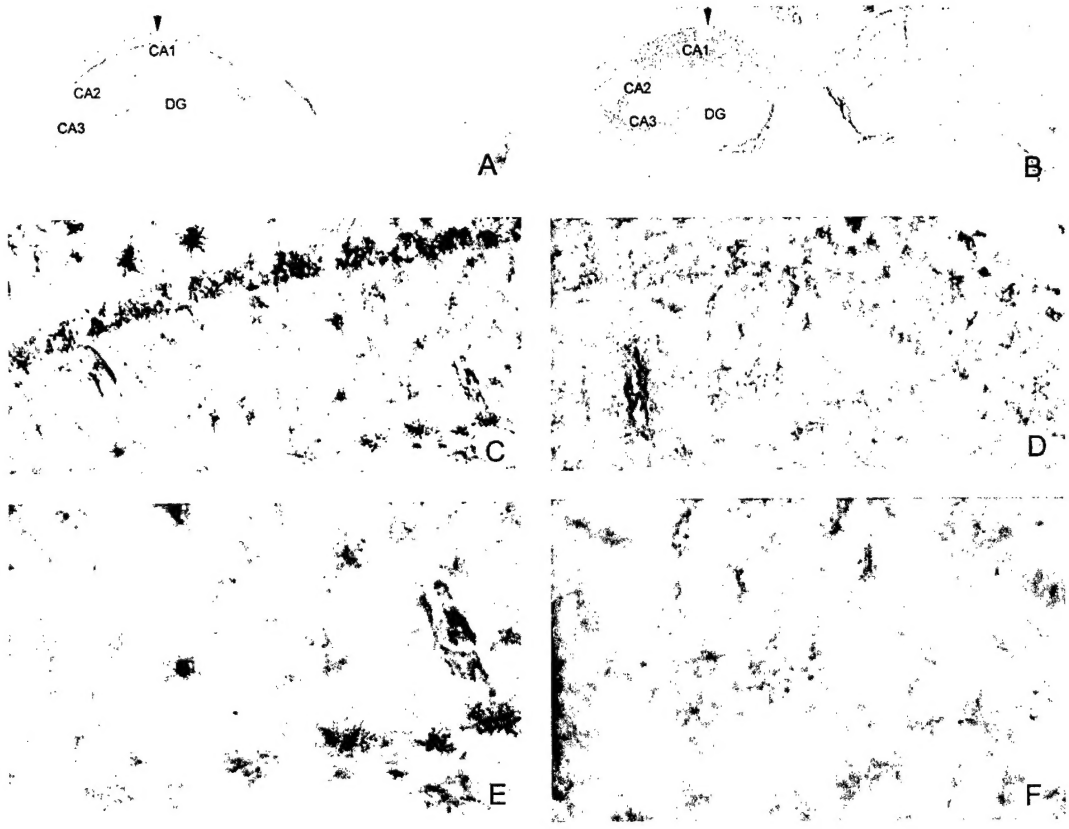
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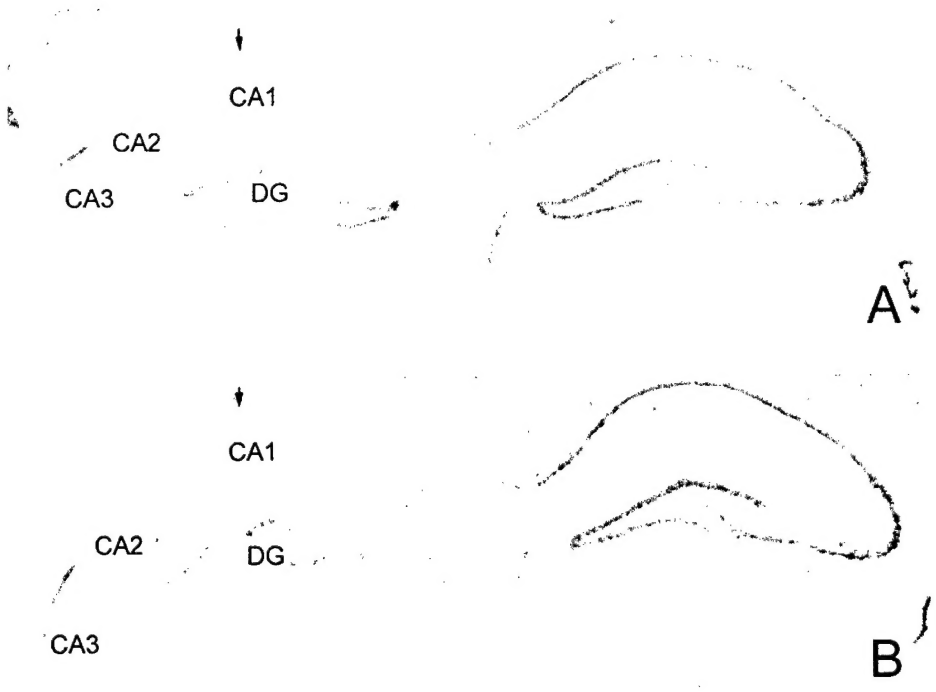
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P326. Kainate-induced neuronal cell death in *op/op* mice points to the presence of a necessary and sufficient number of activated microglia

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Neuronal cell death occurs in the mouse hippocampus following the injection of excitotoxins. This neurodegeneration is accompanied by activation of microglia in the hippocampus. We have previously shown that the pharmacological inhibition of microglial activation can reduce the amount of neuronal death that follows excitotoxin injection into the hippocampus. These data suggested that microglia possess neurotoxic properties. However, it was unclear how potent these cells were after activation. To address this question we utilized the osteopetrotic (op/op) mouse and the intrahippocampal injection of kainate. The basic defect in the op/op mouse is a deficiency in CSF-1 expression. This leads to an inability of microglia to proliferate following CNS injury, although they do exhibit many of the other characteristics of normal activation. The neuronal response to excitotoxin in op/op mice was essentially the same as that observed in control mice, death of the pyramidal cells. As expected, the microglia in op/op did not proliferate in response to kainate injection, but they did exhibit several signs of normal activation, suggesting that the activation of microglia, rather than proliferation is the more important event. Taken together with our previous data on inhibition of microglial activation, it appears that activated microglia secrete very potent neurotoxins when activated, and that even few microglia are enough to effect excitotoxin-mediated neurodegeneration. These data have implications for potential neuroprotective therapies that involve inhibition of microglial activation. It would seem that such therapies would have to totally inhibit microglial activation to be maximally effective.

P327. Effect of Manganese intoxication on the fine structure of neuroglia of the rat's brain

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A number of recent investigations suggest that glial cell activation play important pathogenic roles in the development of neurodegenerative disorders. On the other hand it is well known that overdosage of Manganese produces a neurological symptoms similar to Parkinson's disease. The majority of pathomorphological observations concerning experimental manganese intoxication were carried out at the light microscopic level. We examined the effects of one month peroral administration of $MnCl_2 \cdot 4H_2O$ (50 mg/kg – every day) on fine structure of glial cells of the fronto-parietal cortex, caudate nucleus and substantia nigra of rat's brain. The experimental rats were divided into two groops: I group of animals was killed one day after of manganese chloride last administration and II group of animals-3 months later. In areas studied degeneration of some neurons and synapses, as well as glial reaction was seen. The general pattern of ultrastructural changes of neuroglia was similar in all investigated regions; although their intensity was greatest in the substantia nigra. In astrocytes profiles of vacuolar system and mitochondria were swollen, cristae were disorganized; perivascular and perineuronal processes of astrocytes were swollen and accumulation of glycogen granules was seen. In some astrocytes the accumulation of dense bodies was appear. Oligodendroglial reaction was less manifested – there was only disorganization of some mitochondria. The activity of microglia was sharply increased – there was accumulation of residual bodies and secondary lysosomes, disorganization of mitochondria and vacuolar system. Sometimes the reactive microgliocytes was separated from adjacent neurons by thin lamellar astrocytic processes. Three months after the termination of manganese intoxication degenerative changes in neurons, as well as alterations in astrocytes is still appear; at the same time the number of reactive microgliocytes increases. We conclude that above described alterations were a result of both – direct influence of manganese on the neuroglia and secondary glial reaction caused by neuronal degeneration.

P328. PrP induced interleukin-6 synthesis by adult human microglia is not affected by COX-inhibitors

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In transmissible spongiform encephalopathies (prion diseases) neurodegeneration is thought to be mediated through the effects of microglia activated by the protease resistent isoform of the prion protein (PrP). Interleukin-6 (Il-6), IL-1 and prostaglandin E2 (PGE2) co-localize in PrP accumulations in murine scrapie. Previously we reported that in human astrocytes IL-1ß induced IL-6 synthesis is, in part, mediated through prostaglandin E2. To investigate 1) if synthetic peptides, representing the neurotoxic fragment of the prion protein (PrP) can induce the synthesis of inflammatory mediators by adult human microglia *in vitro* 2) whether microglial IL-6 synthesis is prostaglandin E2 mediated. Mouse $PrP_{105-132}$ that includes the transmembrane part of PrP and is fibrillar as judged by electron microscopy, stimulated microglial IL-6 and, to a lesser extent, TNF-α synthesis. The amidated, nonfibrillar form of $PrP_{105-132}$ only had minor effect, suggesting importance of fibrillarity for the PrP effects. Scrambled $PrP_{105-132}$ had no effect. No induction of IL-1ß synthesis (as determined by ELISA) was observed upon 24 or 72 hrs of culture in the presence of $PrP_{105-132}$. Baseline levels of secreted PGE2 (measured by ELISA) varied among microglial cultures from different cases. LPS clearly stimulated PGE2 secretion. No significant stimulatory effect of $PrP_{105-132}$ on PGE2 synthesis was observed. Indomethacin, a non-selective cyclooxygenase (COX) inhibitor, as well as BF389, a COX-2 selective inhibitor, tended to lower the microglial PGE2 synthesis, whereas they did not exert inhibitory effects on PrP induced IL-6 synthesis by microglia. Conclusion: PrP peptides can stimulate adult human microglial cells to secrete IL-6 and TNF-α in vitro. The apparent inability of COX inhibitors to abrogate the PrP-induced IL-6 synthesis suggests that the microglial PrP-induced IL-6 synthesis is not PGE2 mediated.

P329. Activation of human microglial cells by PrP fragments and induction of nitric oxide synthase

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Activated microglia is involved with the pathogenesis of degenerative disorders of the central nervous system such as Alzheimer's disease and Creutzfeldt-Jacob disease. To gain further insight into the mechanism of microglia activation, we used a pure culture of human resting microglia as a model system. Treatment of these cultures with the fragment 106-126 of the prion protein (PrP) induces a dose-dependent increase in cell proliferation and a shift from ramified to ameboid morphology. Microglial activation is associated to an increase in intracellular calcium concentration and it is abolished by inhibitors of L-type voltage-sensitive calcium channels. Besides, treatment of these cells with PrP106-126 increases the mRNA expression of the inducible nitric oxide synthase (iNOS) while in control cells treated with the scrambled peptide the level of the mRNA for iNOS is identical to the one observed in untreated cells. The iNOS induction by PrP106-126 can be suppressed by the administration of NO donors such as NOR-3 and SIN-1. Since we have previously demonstrated that in microglial cells NO can regulate the transcription of iNOS by acting through the transcription factor NF-kB, the involvement of NF-kB, STATs and SP-1 in the transcriptional regulation of iNOS in PrP-treated microglia is analysed. Uncontrolled and massive iNOS-induced NO production is potentially detrimental to tissue integrity. Moreover, it has been reported that iNOS mRNA levels are increased in scrapie-infected mice compared to the levels in non-infected mice of the same age suggesting that iNOS induction is a part of the neurodegenerative mechanisms in prion diseases. Acknowledgements: This work was supported by MURST 1997

id electrophysiologically on retinal Müller lial cells (MC) of various species, but only few ata exist about its efficiency. In the present udy we used freshly dissociated MC of guinea pig retina to investigate the action of GABA on MC by electrophysiology and by NAD(P)H-imaging. For the cellular and subcellular localisation of the GABA-transporters (GAT) and the GABA-degrading enzyme (GABA-T), isolated cells and retina sections were stained with antibodies. Electrophysiological recordings showed that GABA-evoked currents depend on the membrane potential and GABA-concentration. The affinity constant shifts from 17μM at -120mV to 2μM at 0mV, where the maximal current amplitude decreases from 37 to 5pA. Since the currents were insensitive to picrotoxin and saclofen, the expression of GABA receptors can be excluded. Replacement of extracellular Na^+ or Cl^- inhibits the GABA-evoked current. Therefore the observed current must be due to an electrogenic GABA-transporter. Immunhistochemical studies using antibodies against different GAT showed intense staining of MC for GAT-3 and a weak signal for GAT-1. When GABA enters the cell it is converted to glutamate by the mitochondrial enzyme GABA-T by a NAD(P)-dependent process. The reduction of NAD(P) to NAD(P)H is measureable by changes in autofluorescence. Application of GABA to MC led to an increase of the fluorescence at the proximal region. Using an antibody against GABA-T this part of the cell shows an intense staining. In conclusion, GABA released by retinal neurons is rapidly removed from extracellular space and metabolised by adjacent MC. This may underline their important role for normal GABAergic transmission in the mammalian retina.

P240. Interferon-γ affects calcium homeostasis in primary hippocampal astrocytes

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Calcium oscillations and waves, occurring in cultured astrocytes and in brain slices, are thought to be a form of glial excitability which represents an information processing system operating in parallell with neuronal circuits. Early in the course of a number of pathological conditions of the central nervous system, cytokines are produced by both resident glia and by invading cells of the immune system. In this study we investigated whether inflammatory cytokines may affect calcium signaling in astrocytes, possibly altering information processing. Through their action on astrocytes, cytokines may indirectly compromise neuronal function and, indeed, it is now widely believed that an efficient synaptic functionality essentially requires the participation of adjacent astrocytes. We have examined the effect of the proinflammatory cytokine INF-γ on spontaneous calcium oscillations in primary cultures of hippocampal astrocytes and neurons. Whereas application of IFN-γ does not alter intracellular calcium homeostasis in neurons maintained in pure neuronal cultures, application of INF-γ to cultured astrocytes induces elevations in $[Ca^{2+}]i$, either increasing the frequency or triggering the occurrence of $[Ca^{2+}]i$ oscillations (average increase in the oscillation frequency: 490%). The INF-γ-induced $[Ca^{2+}]i$ oscillations in astrocytes often results in $[Ca^{2+}]i$ elevations in neighboring hippocampal neurons growing in cocultures of neurons and astrocytes. These results indicate that INF-γ affects calcium signaling in astrocytes, altering therefore the bidirectional communication between neurons and astrocytes.

P241. ATP-Sensitive potassium channel regulates astrocytic gap junction permeability. A link between energy metabolism and cell-cell communication

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The permeability of astrocytic gap junctions is subject to regulation and plays an important role in brain metabolism and development. It has been proposed that changes in the membrane potential lead to modifications in the open/closed state of gap junction channels. Thus, membrane depolarization promotes an increase in gap junctional permeability in astrocytes while membrane hyperpolarization reduces the permeability through gap junctions. One of the mechanisms involved in maintaining membrane potential is the activity of the ATP-sensitive potassium channel (KATP). When the KATP channel is inhibited by ATP or sulfonylureas, the concentration of K^+ increases intracellularly leading to plasma membrane depolarization. In addition, ADP and several other agents, the so-called potassium channel openers (KCOs), are able to activate the KATP channel. In contrast to sulfonylureas, KCOs promote hyperpolarization of the plasma membrane. Using the scrape-loading technique in cultured astrocytes we show that sulfonylureas such as tolbutamide and glybenzcyclamide, which inhibit the ATP-sensitive K^+ channel, prevent the inhibition of gap junction permeability caused by several structurally non-related uncouplers such as, oleic acid, arachidonic acid, endothelin-1, octanol or α-glycyrrhetinic acid. When the intracellular level of Ca^{2+} was diminished, all the uncouplers tested were still able to inhibit gap junction communication, indicating that their inhibitory effect was not mediated by Ca^{2+}. In addition, tolbutamide and glybenzcyclamide prevented the inhibitory effect of these uncouplers in Ca^{2+}-depleted astrocytes, suggesting that the inhibition of the ATP-sensitive K^+ channel increases gap junction permeability through a Ca^{2+}-independent mechanism. The activation of the ATP-sensitive K^+ channel caused by potassium channel openers such as diazoxide and pinacidil led to the inhibition of gap junction communication and overcame the effect of sulfonylureas. These results suggest that the ATP-sensitive K^+ channel regulates gap junctional permeability.

P242. Neuronal- and microglial-specific tissue plasminogen activator play different but complementary roles in excitotoxicity-induced neurodegeneration in the mouse

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Microglia, considered the immune cells of the brain, play a critical role in the neurodegeneration seen after excitotoxicity. During excitotoxic injury induced by kainic acid (KA) injection into the hippocampus, hippocampal pyramidal neurons first undergo necrotic, then apoptotic death. In order to understand how microglia are involved in this process, we focus on the protease tissue plasminogen activator (tPA). tPA was shown to mediate neuronal degeneration in the hippocampus after mice were injected with KA. In addition, tPA is involved for activating microglia during excitotoxicity. Therefore, we have taken two approaches to understand whether there is a temporal or a source-specific role of tPA in mediating neuronal degeneration and microglial activation. Specifically, we have cultured primary neurons and microglia together, but separated by a porous membrane so that only diffusible molecules can pass through. We show that microglial, but not neuronal, tPA is mostly responsible for killing neurons after glutamate excitation. Furthermore, neuronal and, to a lesser extent, microglial tPA is responsible for microglial activation. To confirm this data, we have generated transgenic mice that express neuronal- or microglial-tPA on a tPA-null background. We subjected these mice to unilateral hippocampal KA injection, and then examined neuronal degeneration and microglial activation. Both neuronal- and microglial-tPA-expressing mice are susceptible to excitotoxic neurodegeneration. The degree of susceptibility is dependent on the amount of tPA secreted by cells.

P243. Olfactory bulb ensheathing cells and Schwann cells interact differently with astrocytes *in vitro*

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The olfactory ensheathing cell (OEC) has become a cell of great interest due to its ability to remyelinate axons in demyelinating lesions and support re-growth of transected axons following transplantation. Schwann cells (SCs) share these repair properties but have limitations imposed on their behaviour by the presence of astrocytes. Since OECs exist alongside astrocytes in the olfactory bulb we have hypothesised that they have advantages over SCs